Associations between TS, TTF-1, FR-α, FPGS, and Overall Survival in Patients with Advanced Non–Small-Cell Lung Cancer Receiving Pemetrexed Plus Carboplatin or Gemcitabine Plus Carboplatin as First-Line Chemotherapy

Bjørn H. Grønberg, MD, PhD,† Marius Lund-Iversen, MD,‡ Erik H. Strøm, MD,‡ Odd Terje Brustugun, MD, PhD,§‖ and Helge Scott, MD, PhD‡§*

Introduction: Pemetrexed is effective in the treatment of non–small-cell lung cancer, mainly in nonsquamous cell carcinomas. Inhibition of thymidylate synthase (TS) is considered the key mechanism of action. Folate receptor-α facilitates uptake of pemetrexed. Polyglutamation by folylpolyglutamate synthetase enhances activity and prolongs cellular retention of pemetrexed. Thyroid transcription factor-1 (TTF-1) is mainly positive in nonsquamous cell carcinoma and has been proposed as a marker for sensitivity to pemetrexed. The aim was to investigate associations between these biomarkers and survival in patients who participated in a phase III trial comparing pemetrexed plus carboplatin with gemcitabine plus carboplatin as first-line chemotherapy in advanced non–small-cell lung cancer (n = 436). In this study, there was no difference in overall survival between the two regimens.

Methods: Formalin-fixed, paraffin-embedded biopsies were collected. Percentages of tumor cells positive and highly positive for the biomarkers were assessed using immunohistochemistry (IHC) and an IHC score was calculated (range, 0–200).

Results: Two hundred thirty-six biopsies were analyzed (pemetrexed plus carboplatin: n = 114, gemcitabine plus carboplatin: n = 122). There was a significant difference in overall survival between those with TTF-1–positive and –negative tumors (10.4 versus 6.0 months; $p < 0.001$) and those with a low and a high TS IHC score (9.7 versus 6.2 months; $p < 0.001$). Folate receptor-α and folylpolyglutamate synthetase were not significant prognostic factors. In multivariate analyses adjusting for established prognostic characteristics, TS ($p = 0.002$) and TTF-1 ($p = 0.003$) remained significant. There were no differences in survival between the treatment arms depending on biomarker scores.

Conclusions: TTF-1 positivity and low TS level were associated with prolonged survival. The associations between the biomarkers and overall survival were similar for both chemotherapy regimens.

Key Words: Non-small-cell lung cancer, Biomarkers, Pemetrexed, Gemcitabine, Survival, Thyroid transcription factor-1, Thymidylate synthase, Folylpolyglutamate synthetase, Folate receptor.

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Platinum-doublet chemotherapy remains the standard treatment for most patients with advanced non–small-cell lung cancer (NSCLC).[1] However, the survival benefit is limited, and many experience severe side effects.[2] Identifying biomarkers that are associated with outcomes of specific regimens would help improve efficacy and avoid ineffective, potentially harmful therapy.

Pemetrexed is a multitargeted antifolate that inhibits three enzymes in the folate pathway involved in nucleotide synthesis; thymidylate synthase (TS), dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase. Studies have demonstrated that pemetrexed is effective as first-line,[3,4] second-line,[5] and maintenance therapy of NSCLC.[6,7] Subgroup analyses have revealed that the agent is mainly effective and superior to other regimens in nonsquamous cell carcinomas (non-SCCs).[6,8]

Inhibition of TS is thought to be the main mechanism of action.[9] In cell lines from colon and lung cancer, resistance to pemetrexed was associated with TS overexpression.[10–13] In patients with malignant mesothelioma,[14,15] breast cancer,[16] and NSCLC,[17–20] low TS levels were associated with a better response to pemetrexed. Others have found that SCCs have higher TS levels than non-SCCs and that TS levels in small-cell lung cancer are higher than in NSCLC.[21–23] It has been

*The Cancer Clinic, St. Olavs Hospital—Trondheim University Hospital, Trondheim, Norway; †European Palliative Care Research Centre, Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; Departments of ‡Pathology, ‖Oncology, Oslo University Hospital, Oslo, Norway; and §Institute of Clinical Medicine, University of Oslo, Oslo, Norway.

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Address for correspondence: Bjørn H. Grønberg, MD, PhD, European Palliative Care Research Centre, 3. Etg Bevegelsessenteret, PO Box 3250 Sluppen, 7006 Trondheim, Norway. E-mail: bjorn.h.gronberg@gmail.com

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hypothesized that this explains why non-SCCs are more sensitive to pemetrexed than SCCs,[21,22] and why most small-cell lung cancers are resistant to pemetrexed.[24,25]

Folate receptor-α (FR-α) mediates cellular uptake of folate essential for synthesis of RNA and DNA, may facilitate transport of pemetrexed into cells, and has been proposed as a biomarker for antifolate therapy.[26,27] Folylpolyglutamate synthetase (FPGS) activates pemetrexed via polyglutamation and prolongs its cellular retention. Low levels of FPGS may thereby decrease the antitumor activity. A study of leukemia cells suggested that low FPGS level was associated with resistance to pemetrexed.[28]

Thyroid transcription factor-1 (TTF-1) is mainly expressed in non-SCC and is an important marker for subclassification of NSCLC when no clear morphologic features can be found.[29] In one study, TTF-1 positive tumors had higher response rates to pemetrexed than TTF-1 negative,[18] and it has been proposed that the biomarker can explain why pemetrexed is mainly active in non-SCC.

Gemcitabine is one of the standard therapies of NSCLC.[30] It is a pyrimidine-analogue that inhibits DNA synthesis by inducing depletion of cellular deoxynucleotides and through incorporation into DNA.[31]

Our study group conducted a phase III trial comparing pemetrexed plus carboplatin (PC) with gemcitabine plus carboplatin (GC) as first-line chemotherapy in advanced NSCLC.[3] The aim of this study was to investigate associations between TTF-1, TS, FR-α, or FPGS and overall survival in participants of this trial, and to determine whether there were different associations between these biomarkers and overall survival between the two treatment arms.

MATERIALS AND METHODS

The main eligibility criteria for the phase III trial were stage IIIB (ineligible for curative radiotherapy) or stage IV NSCLC; no previous chemotherapy; age ≥18 years; and World Health Organization performance status (PS) 0 to 2. Patients received up to four cycles of carboplatin area under the curve = 5 (Calvert's formula) plus pemetrexed 500 mg/m^2 day 1 (PC) or carboplatin area under the curve = 5 day 1 plus gemcitabine 1000 mg/m^2 days 1 and 8 (GC) every 3 weeks. Four hundred thirty-six eligible patients were enrolled from May 2005 until July 2006 at 35 hospitals in Norway. The survival analyses were finalized in July 2007 after a median observation time of 19 months. The main conclusions were that there were no differences in health-related quality of life or overall survival between the arms. More hematological toxicity was observed on the gemcitabine arm.[3]

Patients were included in the present study if we were able to collect formalin-fixed, paraffin-embedded tumor tissue for immunohistochemicial analyses.

Design and Approval

This retrospective biomarker study was approved by the Regional Committee for Medical Research Ethics, Central Norway; the Norwegian Social Science Data Services; and the Norwegian Directorate for Health and Social Affairs.

Immunohistochemical Assays

Tissue micro arrays were built of one to three cores (1-mm diameter) from the tumor samples when possible. Otherwise, sections were cut from the whole remaining tissue blocks. Sections, cut at 4 µm, were positioned on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany) and

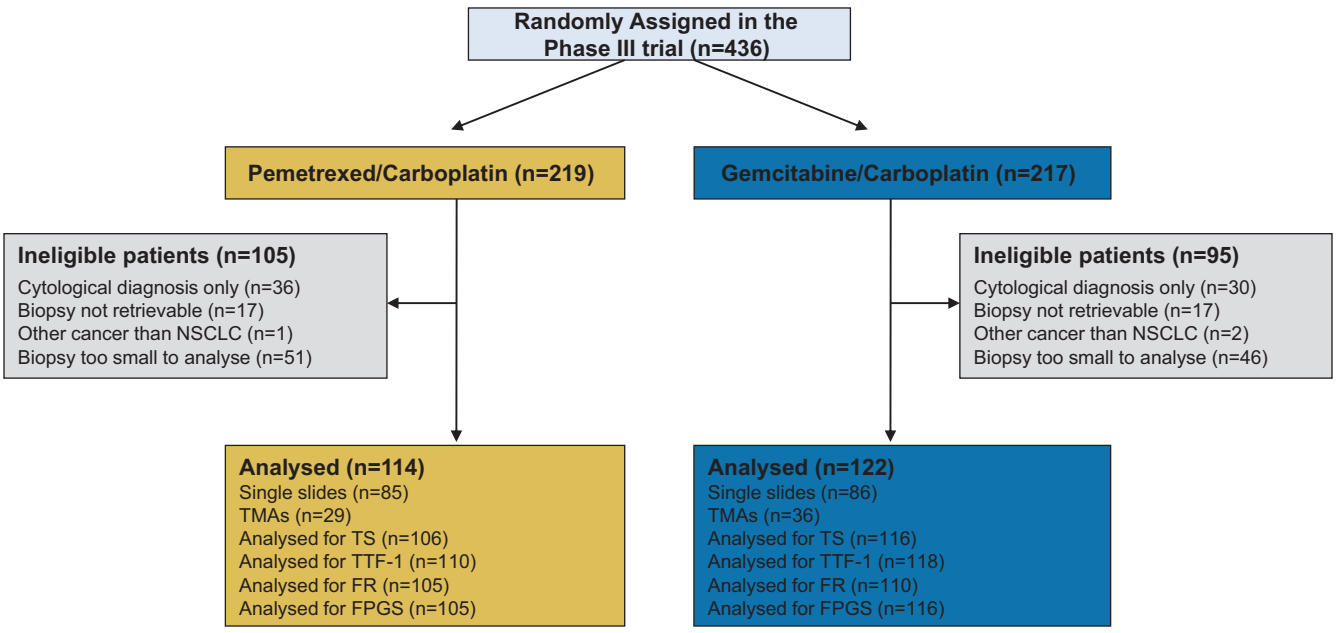

FIGURE 1. Patient selection for the biomarker study. TS, thymidylate synthase; TTF-1, thyroid transcription factor-1; FR, folate receptor; FPGS, folylpolyglutamate synthetase; NSCLC, non–small-cell lung cancer; TMAs, tissue micro arrays.

deparaffinized. Ventana's Bench Mark XT staining platform was used for immunohistochemistry (IHC). Antigen retrieval was conducted under standard conditions with cell conditioning 1 (CC1) buffer (VMSI, Tucson, AZ; Catalog No 950-124). Slides were incubated with the appropriate dilution of the primary antibody for 1 hour at room temperature. The antibodies to TS, FR-α, and FPGS were noncommercial murine monoclonal antibodies from Eli Lilly & Co. (Indianapolis, IN); the monoclonal anti–TTF-1 was from Ventana Medical Systems (VMSI; Catalog No 760–2829). As a negative control, specimens were incubated with mouse immunoglobulin under the same conditions. Sections from a paraffin-embedded, carcinoma specimen, earlier tested and found positive for the markers by Ventana Medical Systems, were used as positive controls—one section for each marker in each run. Binding of primary antibodies to the tissue samples was detected using the Ultra View detection kit (VMSI; Catalog No 760-500). Enzymatic detection was accomplished with a streptavidin horseradish peroxidase conjugate, followed by reaction with hydrogen peroxide in the presence of diamino benzidine and copper sulfate.

Scoring

Three pathologists (MLI, HS, and EHS) scored the slides. All pathologists underwent training by a pathologist from—and scored the slides as recommended by—the company that developed the immunoassays (Dr. Eric Powell; Ventana Medical Systems Inc.). All pathologists individually scored 10% of the samples for all biomarkers. Because of very high interrater reliability, the slides were shared, and the rest of the slides for each marker were scored by one of the three pathologists. An intensity identified as higher than background and specific for the cells of interest was scored as 1+. Staining with an intensity that allowed detection of positive tumor cells at low magnification (40×) was scored as 2+. Percentages of negative (0), positive (1+), and highly positive (2+) tumor cells in each sample were assessed. An IHC score was calculated for each marker on each sample: IHC score = ([% of 1+] × 1) + ([% of 2+] × 2) providing a range of 0 to 200. We were not able to accurately distinguish between FR expression in the cytoplasm and in the membrane. When more than one sample from one biopsy was analyzed, the average IHC score was used in the analyses.

Statistical Considerations

Survival was defined as time from random assignment until death and was estimated using the Kaplan–Meier method. Survival data were compared using the log-rank test. The Cox proportional hazards method was used to calculate hazard ratios (HRs). The ANOVA test was used to compare IHC scores between the different histological subgroups. In the comparative biomarker analyses, we used the median IHC score as a cutoff point. Furthermore, we compared the lower and upper quartiles; tumors with any positive versus all negative cells; and tumors with any highly positive cells versus the others. Interaction tests were performed to investigate whether any combinations of biomarkers were associated with differences in overall survival. Because the use of platinum-doublet chemotherapy is debated in PS 2 patients, all the analyses were also separately conducted for patients with PS of 0 to 1. Statistical significance level was defined as p value less than 0.05.

RESULTS

Patients

The NSCLC diagnosis was confirmed histologically in 370 patients. We were able to retrieve paraffin blocks from 336 of these. In 97 cases, there was not enough tumor tissue for IHC analyses, and in three cases, a central pathology review revealed other cancer than NSCLC. Thus, a total of 236 patients were included in the present study. In 65 cases, there was sufficient tissue to build tissue micro arrays. We were not able to analyze all samples for all four biomarkers;

TABLE 1. Baseline Characteristics of the Patients Included in the Biomarker Study

		Carboplatin/ Pemetrexed (n = 114)		Carboplatin/ Gemcitabine (n = 122)	
		n	%	n	%
Age	Median	62		64	
	Range	35–85		37–81	
Sex	Men	60	53	66	54
	Women	54	47	56	46
Stage	IIIB	36	32	39	32
	IV	78	68	83	68
Performance status	0	28	25	22	18
	1	60	53	68	56
	2	26	23	32	26
Histology	Adenocarcinoma	52	46	60	49
	Squamous cell carcinoma	31	27	31	25
	Large-cell carcinoma	6	5	7	6
	Other	25	22	24	20
Smoking history	Never smoker	11	10	7	6
	Former smoker	61	54	64	53
	Current smoker	42	37	51	42
Cycles of chemotherapy	Mean	3.2		3.1	
Any systemic second-line therapy		39	34	36	30
	Docetaxel	14	12	11	9
	Reinduction	2	2	7	6
	Pemetrexed	2	2	3	3
	Carboplatin/ vinorelbine	4	4	5	4
	Erlotinib	12	11	9	7
	Vinorelbine	2	2	-	-
	Other	3	3	1	1
Any systemic third-line therapy		5	4	8	7

222 were analyzed for TS; 228 for TTF-1; 215 for FR-α; and 221 for FPGS (Fig. 1).

Median age of the patients was 63 years (range, 35–85); 53% were men; 32% had stage IIIB; 75% had PS 0 to 1; 8% were never-smokers; 48% received PC, 52% GC. The baseline characteristics, number of cycles of chemotherapy administered, and the use of poststudy systemic cancer therapy were similar in the two treatment arms (Table 1).

Tumor Samples

The biopsies were collected from the lung or mediastinum in 86% of the patients; from lymph node metastases from the neck or supraclavicular region in 6%; from bone metastases in 4%; from liver, pleura, brain, adrenal gland, or other lymph nodes in the remaining 4%. The biopsy samples were collected through bronchoscopy in 48% of the patients; percutaneous biopsy in 28%; surgery in 20% (approximately half of these patients had primary surgery and were enrolled when they developed advanced disease; in the other cases, a biopsy sample was collected through surgery), and mediastinoscopy in 4%.

Biomarker Status

The distributions of IHC scores are shown in Figure 2. Among the biopsy samples, 47% (111 of 228) was TTF-1 positive; 42% (100 of 228) had 2+ tumor cells; the median IHC score was 0 (SD: 78). Ninety-three percent (219 of 223) was TS positive; 89% (211 of 222) had 2+ tumor cells, and the median IHC score was 134 (SD: 45). Fifty-six percent (131 of 215) was FR-α positive; 17% (41 of 215) had 2+ tumor cells, and the median IHC score was 10 (SD: 40). Ninety-two percent (219 of 221) was FPGS positive; 57% (134 of 222) had 2+ tumor cells, and the median IHC score was 100 (SD: 61).

The percentages of FPGS 2+ and TTF-1 positive tumors were significantly lower among SCCs ($p < 0.001$). Six cases originally diagnosed as SCCs were TTF-1 positive. There were no significant differences in the percentages of TS 1+

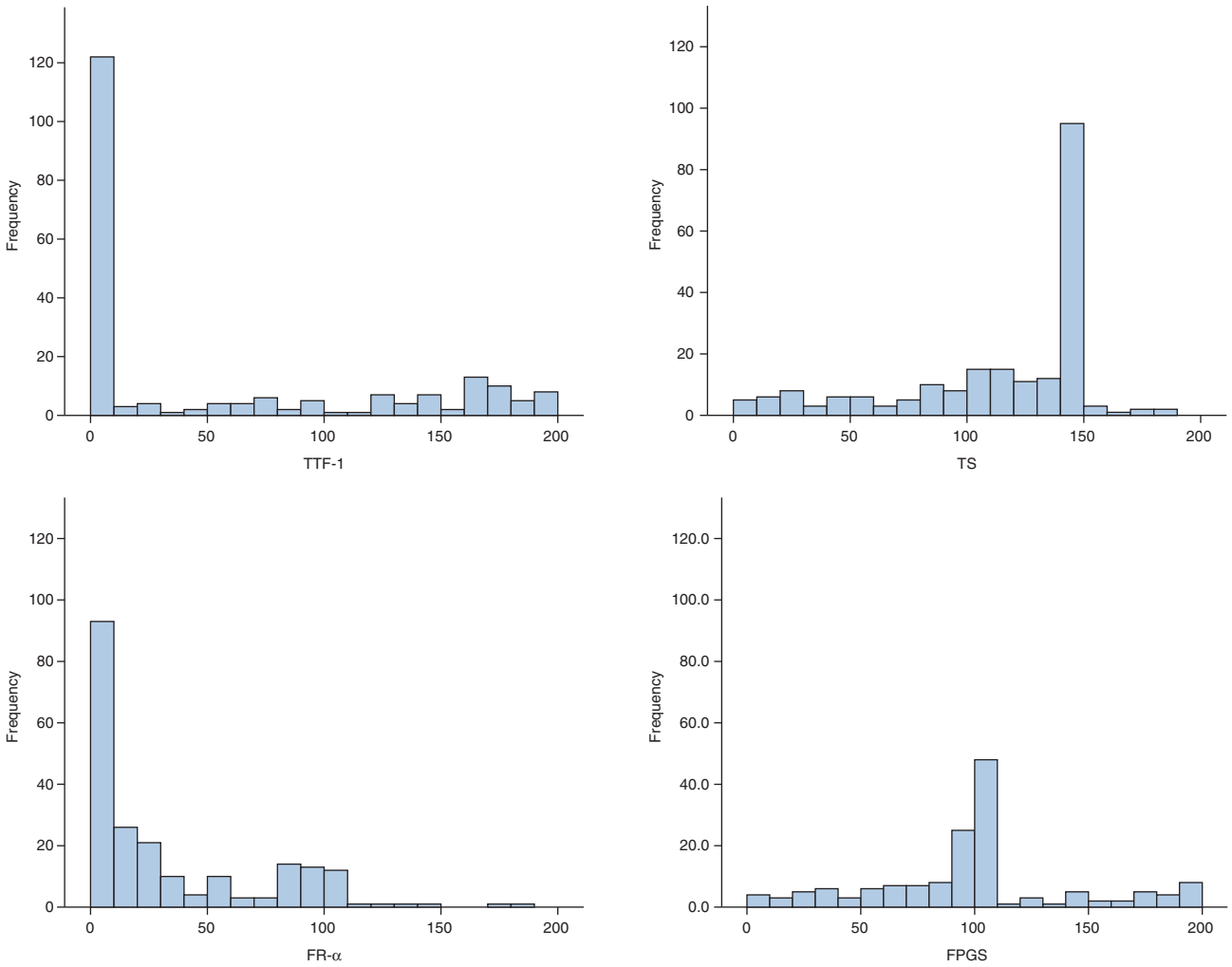

FIGURE 2. Distribution of immunohistochemistry (IHC) scores for all samples. IHC score = (% 1 + positive tumor cells × 1) + (% 2 + positive tumor cells × 2). Range, 0 to 200. TS, thymidylate synthase; TTF-1, thyroid transcription factor-1; FR, folate receptor; FPGS, folylpolyglutamate synthetase.

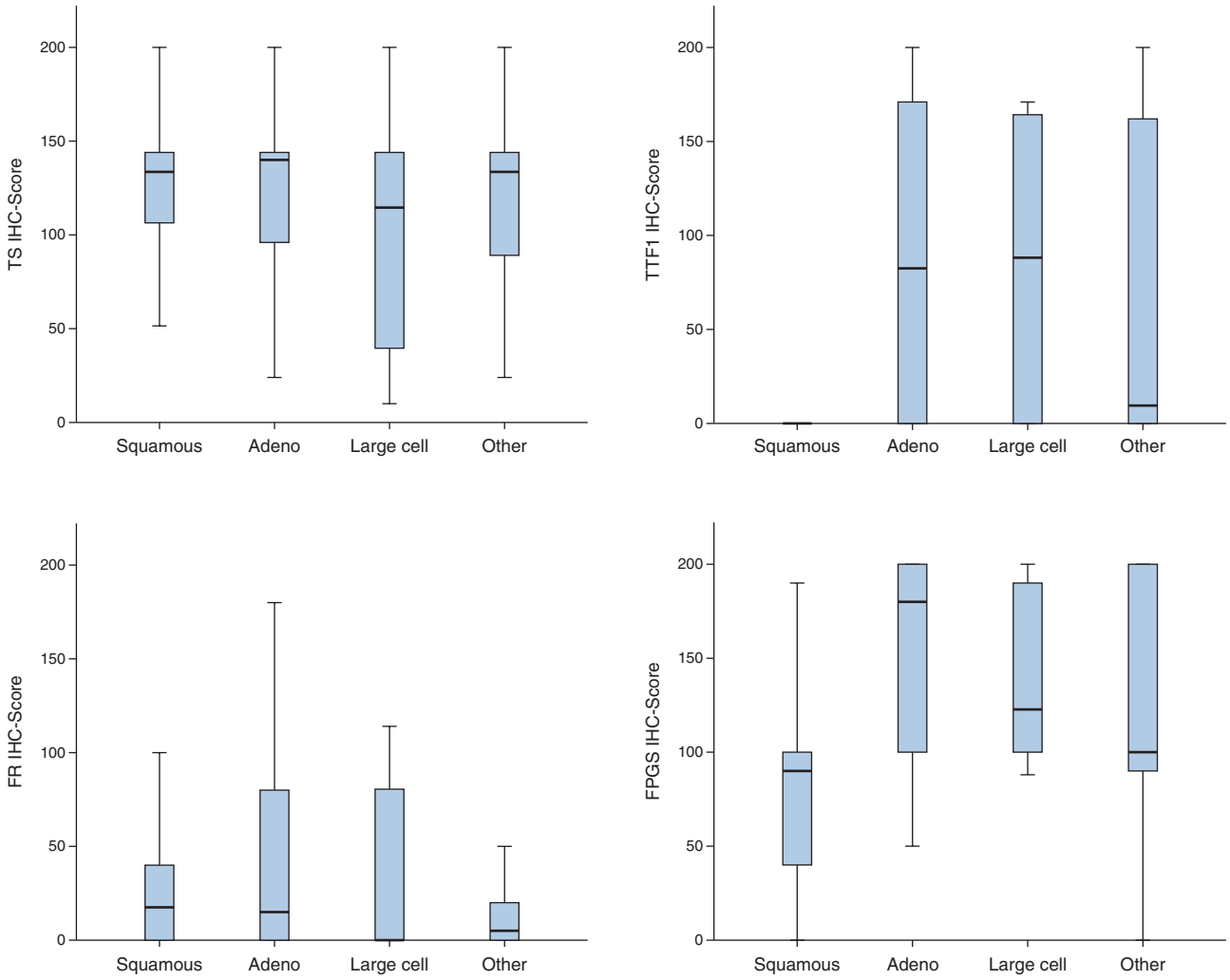

FIGURE 3. IHC scores split for histological subgroups. The box plots show minimum, first quartile, median, third quartile, and maximum values. IHC, immunohistochemistry; TS, thymidylate synthase; TTF-1, thyroid transcription factor-1; FR, folate receptor; FPGS, folylpolyglutamate synthetase.

($p = 0.62$), TS 2+ ($p = 0.35$), FR-α 1+ ($p = 0.16$), FR-α 2+ ($p = 0.07$), or FPGS 1+ ($p = 0.19$) tumors among the histological subgroups.

The median IHC scores for TS ($p = 0.51$) or FR-α ($p = 0.08$) were not different among the histological subgroups, nor were there any differences in TS level when comparing SCCs with adenocarcinomas plus TTF-1–positive large-cell and other carcinomas ($p = 0.30$). The median IHC scores of TTF-1 ($p < 0.001$) and FPGS ($p = 0.001$) were significantly lower in SCCs compared with those of the other subgroups (Fig. 3).

Overall Survival

Results from the univariate survival analyses are shown in Table 2, and a selection of survival curves are shown in Figure 4. Patients with a TS IHC score below the median value had a longer survival than those with a higher TS IHC score (9.7 versus 6.2 months; $p = 0.002$). Those with TTF-1–positive tumors had a significantly longer overall survival than those with TTF-1–negative tumors (10.4 versus 6.0 months; $p < 0.001$). The exclusion of patients with SCC who received pemetrexed did not change the prognostic value of TS or TTF-1 (data not shown). There were no significant differences in survival when comparing those with an FR IHC score over/under the median value (8.4 versus 6.5 months; $p = 0.33$), but there was a trend to prolonged survival for those with an IHC score in the upper quartile when compared with those in the lowest quartile (10.1 versus 6.3 months; $p = 0.075$). There were no significant differences in overall survival depending on FPGS IHC scores. Interaction tests revealed that those with TTF-1–positive tumors and a TS IHC score below the median value had a significantly longer overall survival than the other patients (12.9 versus 6.3 months; $p < 0.001$).

There was no difference in overall survival between SCCs or nonsquamous (adenocarcinomas and large-cell

TABLE 2. Univariate Survival Analyses

		Median (mo)	95% CI		Median (mo)	95 % CI	HR (95 % CI)	*p*
Performance status	0–1 (n = 178)	7.5	5.8–9.1	2 (n = 58)	4.7	3.7–7.7	0.61 (0.45–0.86)	0.004
Stage	IIIB (n = 75)	6.5	5.5–7.6	IV (n = 161)	7.3	6.1–8.6	1.19 (0.83–1.51)	0.47
Sex	Women (n = 110)	7.3	5.9–7.4	Men (n = 126)	6.6	5.4–9.2	0.81 (0.61–1.08)	0.16
Smoking history	Never-smoker (n = 18)	7.3	2.3–12.3	Ever-smoker (n = 218)	6.9	6.1–7.6	0.79 (0.48–1.29)	0.34
Age	<75 yr (n = 197)	6.9	6.0–7.8	≥75 yr (n = 74)	7.1	4.8–9.4	1.03 (0.70–1.53)	0.87
Histology	Squamous cell carcinoma (n = 62)	7.3	5.8–8.7	Adeno- and large-cell carcinoma	7.1	5.3–8.9	1.2 (0.83–1.63)	0.31
Chemotherapy	Pemetrexed/carboplatin (n = 114)	7.1	6.1–8.1	Gemcitabine/carboplatin (n = 122)	6.7	5.4–8.0	0.97 (0.73–1.29)	0.83
	Any second-line therapy (n = 75)	14.2	11.0–17.3	No second-line therapy (n = 161)	5.2	4.3–6.0	0.41 (0.30–0.57)	<0.001
Baseline HRQoL	High global QoL (n = 85)	7.8	5.5–10.0	Low global QoL (n = 151)	6.3	5.5–7.2	0.78 (0.58–1.05)	0.10
	No appetite loss (n = 109)	10.1	8.4–11.9	Appetite loss (n = 127)	5.7	4.8–6.6	0.57 (0.43–0.76)	<0.001
Biomarkers[a]	Low TS (n = 120)	9.7	6.8–12.4	High TS (n = 109)	6.2	5.4–7.1	0.63 (0.46–0.84)	0.002
	TTF-1 positivity (n = 111)	10.4	8.3–12.6	TTF-1 negativity (n = 117)	6.0	4.8–7.2	0.56 (0.42–0.75)	<0.001
	High FR (n = 101)	8.4	6.3–10.5	Low FR (n = 112)	6.5	5.7–7.2	0.85 (0.63–1.14)	0.28
	High FPGS (n = 102)	7.3	5.7–9.0	Low FPGS (n = 119)	6.7	6.0–7.4	0.89 (0.66–1.19)	0.43
	TTF-1 positivity and low TS (n = 66)	12.9	9.0–16.8	Other TTF-1 and TS level (n = 163)	6.3	5.5–7.1	0.71 (0.60–0.84)	<0.001

[a]High was defined as ≥ median IHC score, low as < median IHC score for each marker.

CI, confidence interval; HR, hazard ratio; HRQoL, health related quality of life; TS, thymidylate synthase; TTF-1, thyroid transcription factor-1; FR-α, folate receptor-α; FPGS, folylpolyglutamate synthetase.

carcinomas) in the overall population (7.3 versus 7.1 months; $p = 0.31$); among those who received pemetrexed (n = 91; 7.4 versus 6.8 months; $p = 0.64$); those who received gemcitabine (n = 99; 6.5 versus 7.1 months; $p = 0.32$); among those with a TS IHC score that was equal to or more than the median value (n = 97; 6.6 versus 5.0 months; $p = 0.64$); TS less than median (n = 88; 9.4 versus 11.0; $p = 0.32$); TTF-1 positive (n = 88; 9.4 versus 10.8 months; $p = 0.87$); TTF-1 negative (n = 97; 7.0 versus 5.0 months; $p = 0.20$); FR ≥ median (n = 89; 7.4 versus 8.8 months; $p = 0.20$); FR less than median (n = 89; 6.7 versus 6.4; $p = 0.95$); FPGS equal to or more than median (n = 83; 7.2 versus 7.4 months; $p = 0.09$); or FPGS less than median (n = 95; 7.0 versus 6.8; $p = 0.77$).

PS, use of second-line systemic therapy, and appetite loss at baseline were significant prognostic factors in the univariate analyses. Multivariate analyses adjusting for these factors as well as the other significant prognostic characteristics in the main study (stage of disease, sex, global quality of life, and appetite loss at baseline)[3,32] revealed that use of second-line therapy (HR = 0.48; 95% confidence interval [CI], 0.34–0.69), appetite loss (HR = 0.63; 95% CI, 0.46–0.86), TTF-1 (HR = 0.71; 95% CI, 0.52–0.97), and TS IHC scores (HR = 0.59; 95% CI, 0.44–0.80) remained significant prognostic factors. When including the interaction tests, TTF-1 positivity/low TS turned out to be the strongest prognostic biomarker (HR = 0.68; 95% CI, 0.57–0.81).

There were no differences in overall survival between the treatment arms among those with a low (HR = 1.01; 95% CI, 0.64–1.57) or high (HR = 0.93; 95% CI, 0.64–1.38) TS IHC score; those with TTF-1–positive (HR = 1.10; 95% CI, 0.71–1.69) or –negative tumors (HR = 0.85; 95% CI, 0.58–1.25); high (HR = 0.82; 95% CI, 0.53–1.23) or low (HR = 1.15; 95% CI, 0.76–1.73) FR-α-score or high (HR = 0.82; 95% CI, 0.53–1.23) or low (HR = 1.07; 95% CI, 0.72–1.59) FPGS-score (Fig. 5). Nor were there any different associations between these biomarkers and overall survival between the treatments arms when using other cutpoints for the IHC analyses, in interaction tests, when analyzing patients with PS 0 to 1 separately or when analyzing those who never received any systemic cancer therapy after completing the study treatment.

DISCUSSION

Low tumor TS level and TTF-1-positive tumors were significant positive prognostic factors for survival in our study population of patients with advanced NSCLC. Interaction tests revealed that the combination of low TS and TT1 positivity was the strongest prognostic biomarker score. FR and FPGS were not significant prognostic factors, and there were no differences in associations between the four biomarkers and overall survival between the two treatment arms.

Patients with low tumor TS level had a longer survival than those with a high tumor TS level in the PC arm. But this was also the case in the GC arm, and there were no differences in survival between the treatment arms depending on any TS scores. Possible explanations are that TS is a prognostic factor for survival in NSCLC in general, in patients with advanced NSCLC receiving chemotherapy, or that TS level predicts sensitivity to both of the regimens administered in our trial.

Our results are in accordance with several other reports. Low TS has been associated with lower recurrence rate, longer disease-free survival, or longer overall survival in resected NSCLC[31,33–38] and with better outcomes of first-, second-, and third-line pemetrexed therapy in studies of NSCLC,[17–20] breast

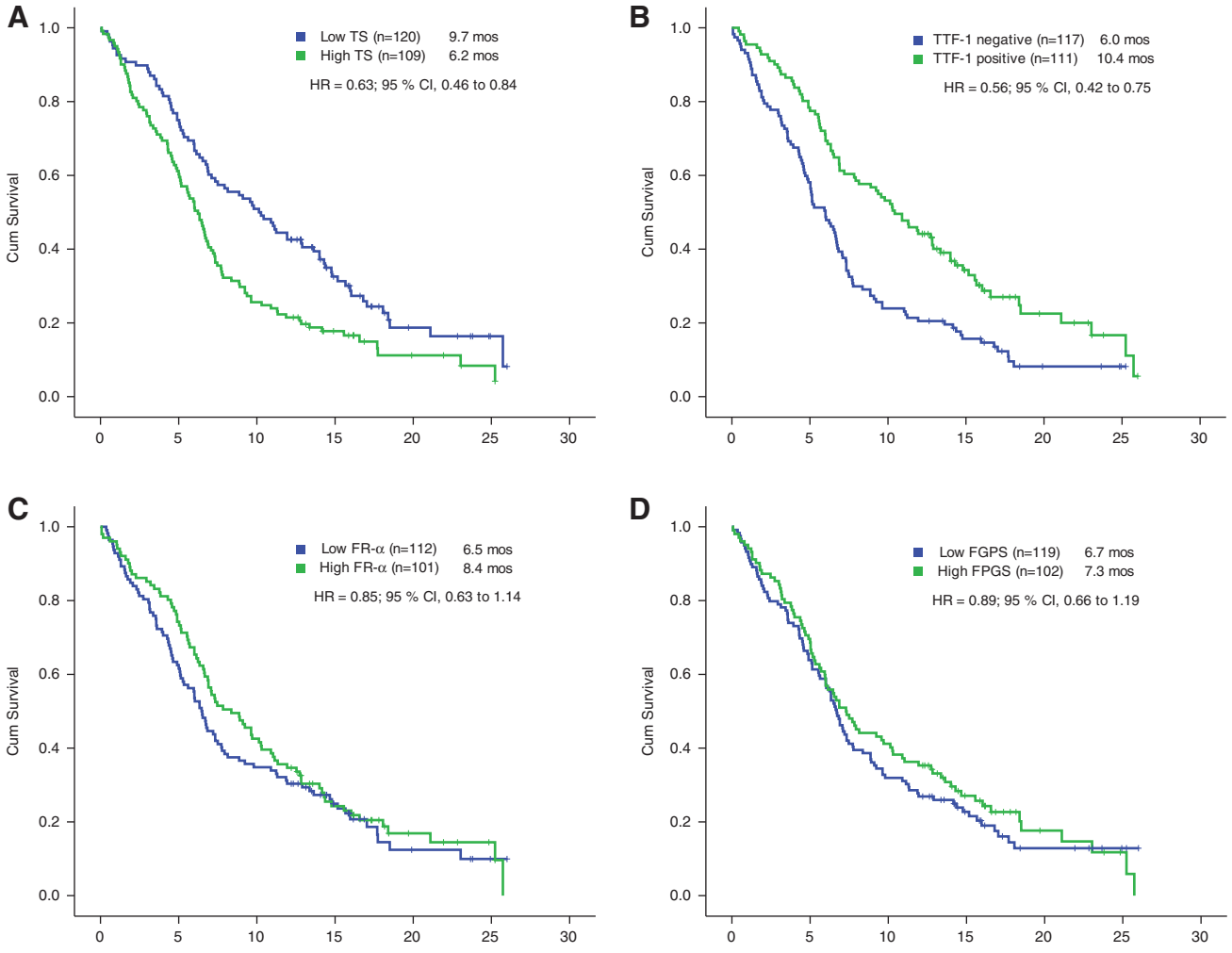

FIGURE 4. Survival depending on IHC scores in the whole study population. *A*, Low versus high TS. *B*, TTF-1 negativity versus positivity. *C*, Low versus high FR-α. *D*, Low versus high FPGS. The median IHC score was defined as the cutpoint for TS, FR-α, and FPGS. TS, thymidylate synthase; TTF-1, thyroid transcription factor-1; FR-α, folate receptor-α; FPGS, folylpolyglutamate synthetase; IHC, immunohistochemistry; HR, hazard ratio.

cancer,[16] and malignant pleural mesothelioma.[14,15] There are, however, a few exceptions. In one study, TS was not a significant prognostic factor in NSCLC,[39] and there was no difference in progression-free survival or overall survival depending on TS level in a study of NSCLC patients who received pemetrexed as third- or fourth-line therapy.[40]

Three studies have investigated whether TS predicts response to gemcitabine. There were no associations between TS level and outcomes of gemcitabine- or taxane-based chemotherapy in a study of advanced NSCLC.[18] In a study of pancreatic cancer, TS was associated with resistance to gemcitabine.[41] In a study of resectable NSCLC, higher response rates were observed among those with a low TS level. But in this study, gemcitabine was administered in combination with pemetrexed.[42] A possible explanation for these observations and the results of our study is that high TS might be correlated to a high proliferation rate,[33,43] and that this influences survival more than the differences in mechanisms of action of pemetrexed and gemcitabine.

Results from previous studies of the prognostic value of TTF-1 are not uniform, but a meta-analysis as well as a recent article conclude that TTF-1 is an independent prognostic factor in NSCLC.[44,45] The associations between TTF-1 and better outcomes of chemotherapy have been observed in NSCLC patients receiving pemetrexed and gemcitabine but not taxane therapy.[18]

There are less data on the role of FR and FPGS in NSCLC. High expression of FR-α was found to be a positive prognostic factor in three of four studies of NSCLC.[20,46–48] FR-α level was not associated with outcomes of pemetrexed therapy in a study of malignant pleural mesotheliomas.[49] There were no significant associations between FPGS and survival among pemetrexed-treated NSCLC patients.[50]

The main strength of our study is that we have compared the associations between the biomarkers and survival in two equally large, well-balanced cohorts that received either a pemetrexed or gemcitabine doublet, providing

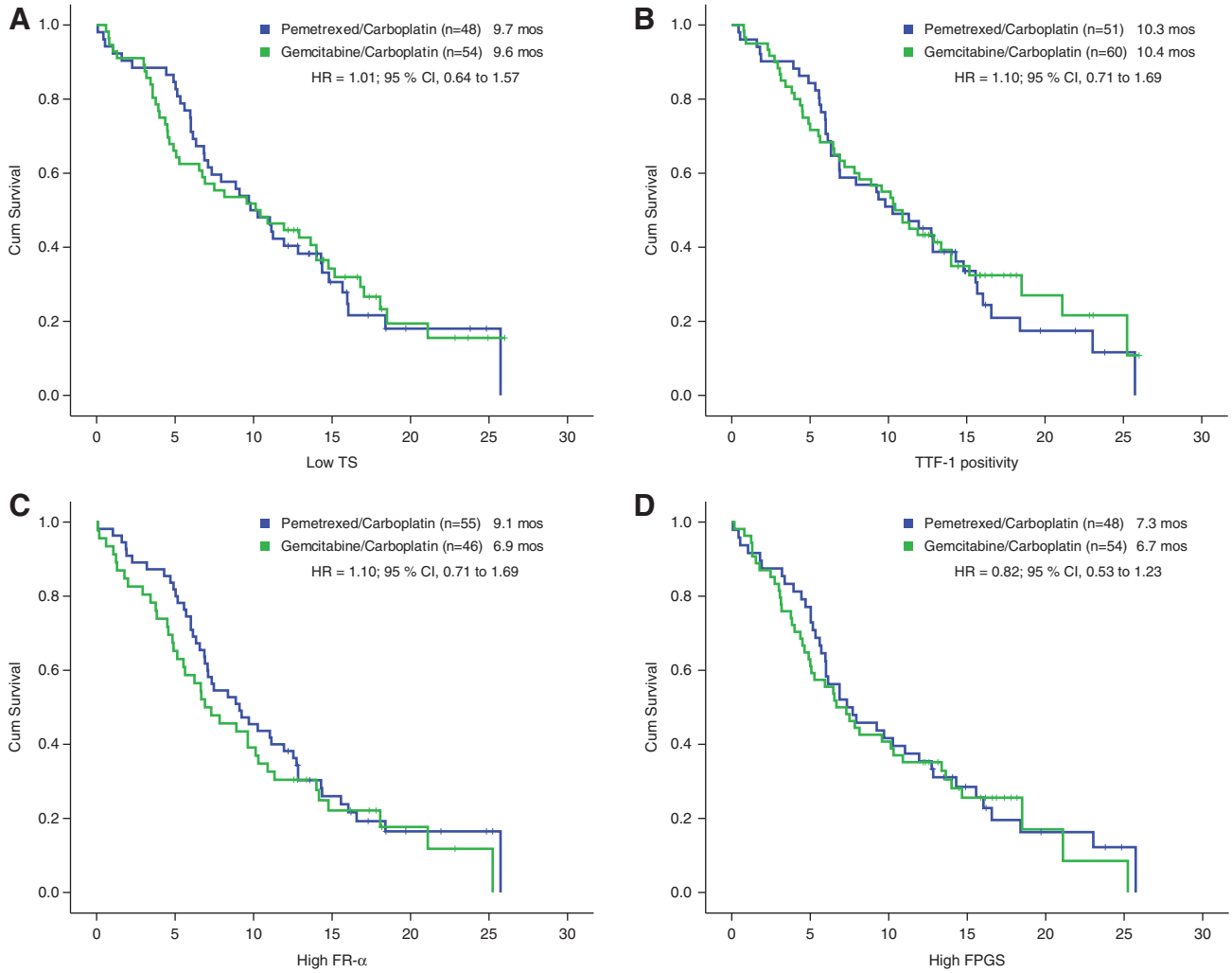

FIGURE 5. Comparison of survival between the treatment arms in cohorts selected through biomarker status. *A*, Patients with low TS. *B*, Patients with TTF-1 positivity. *C*, Patients with high FR-α. *D*, Patients with high FPGS. The median IHC score was defined as the cutpoint for TS, FR-α, and FPGS. TS, thymidylate synthase; TTF-1, thyroid transcription factor-1; FR-α, folate receptor-α; FPGS, folylpolyglutamate synthetase; IHC, immunohistochemistry; HR, hazard ratio.

valuable additional information to the studies that have included pemetrexed-treated patients only. Only three of the patients on the gemcitabine arm (2%) received pemetrexed as second-line therapy, and it is unlikely that this has influenced the analyses.

There are several limitations to our study. It is a retrospective study and we were only able to analyze samples from 54% of the participants in the phase III trial. Because of the design of the original study, we do not have data on response rates or progression-free survival. Thus, we have not been able to investigate whether any of the biomarkers predict sensitivity to either regimen, though we would expect to see different associations between the biomarkers and overall survival between the two regimens if this was the case because two of three of the patients never received any systemic cancer therapy beyond the study treatment.

At the time we conducted the phase III trial (2005–2006), the association between pemetrexed and histology was not known and there was less interest in subclassification of NSCLC. IHC for subclassification of NSCLC had only been performed in 56% of the cases included in this biomarker study, and there was not enough remaining tissue for a comprehensive, central pathologic review (or to test for EGFR mutations, ALK translocations or to compare different antibodies for the immunohistochemical analyses). Through a review of the original slides (by MLI, EHS, and HS), it was not possible to assess the histological diagnoses with certainty in all cases. Thus, we have used the original histological diagnoses in this study. The suboptimal histological classification, in our opinion, best explains why we did not find the differences in TS or FR level depending on histologic findings reported in other studies[22,34,47] though we cannot rule out that this can be because of the choice of antibodies. We have not been able to compare antibodies because of small tumor samples. Suboptimal histological classification can also very well explain why we did not find a pemetrexed/

histology effect in our phase III trial[3] or in the subcohort analyzed in the present study, which is in contrast to several other reports revealing that pemetrexed is mainly effective in nonsquamous NSCLC.[6,8]

Most of the available tumor samples were small. Analyses of the few surgical resection specimens in our cohort revealed that immune-enzyme staining was not evenly expressed by all tumor cells. In such relatively large tissue samples, there are usually areas with necrosis, differences in growth pattern, cell density, and mitotic activity as well as different levels of fixation throughout the sample. All these factors may influence the staining intensity of an antibody. Thus, the expression of biomarkers in biopsy samples might not be representative for the whole tumor or all lesions. But in routine clinical practice, only small biopsy samples are available from a large proportion of patients with advanced NSCLC. Furthermore, Herpel et al.[51] found a good correlation between TS expression in biopsied samples and corresponding resection specimens.

Several methods for assessment of tumor TS level (mRNA, gene copy number, and IHC using different antibodies) have been used in previous studies of TS and outcomes of pemetrexed therapy in NSCLC.[17–20] Because the results of most of these studies are consistent, there is no reason to believe that any method is superior. IHC was chosen in this study because it is a quick method, does not require fresh tumor samples, and thus, is more feasible in routine clinical practice. IHC is, however, limited by subjectivity because it relies on the individual pathologist's interpretation of the slides. In most of the cited studies using IHC, an H-score was calculated to assess biomarker expression. Because of small biopsy samples, we used a modified scoring system using one cutpoint for positivity instead of two because this resulted in far superior interinvestigator agreement. To our knowledge, it has not been demonstrated that H-scores are better correlated to survival in NSCLC than other scoring methods for the four biomarkers we used. Our approach is supported by Herpel et al.[51] who found that the best correlation regarding TS level between biopsied samples and surgical specimens was when only one cutoff point for positivity was used, and a similar, modified IHC score was used in the study by Sun et al.[18]

In conclusion, we found that TS and TTF-1 and TS and TTF-1 combined were significant prognostic factors for survival in patients with advanced NSCLC who received either PC or GC as first-line chemotherapy. The associations between the TS, TTF-1, FR, and FPGS and overall survival were similar for both chemotherapy regimens.

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